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EFFECTS OF METHEMOGLOBIN VERSUS POTASSIUM CYANIDE INTOXICATION

Final Report

WILLIAM D. JOHNSON, PH.D.

August 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701-5012

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Studies were conducted in Beagle dogs to evaluate the efficacy of selected compounds as to potential antidotes against cyanide intoxication in an attempt to develop a drug or regimen of drugs which can be used for protection against cyanide intoxication in a battlefield situation where the threat of cyanide exposure is a likely possibility. The majority of the research centered around measuring the degree of protection from lethal intravenous doses of potassium cyanide (KCN) achieved primarily by increasing blood methemoglobin levels. Initially, the acute LD50 of intravenously administered potassium cyanide was determined. In addition, the chemical method for determining methemoglobin in the blood of dogs was validated and the kinetics of methemoglobin formation and disappearance following administration of WR 6026, an 8-aminoquinoline experimental anti-leishmanial drug, was evaluated. Next, studies were conducted investigating the effect of pre-existing levels of methemoglobin induced by WR 6026 on the ability of the dog to resist cyanide intoxication. Protective indexes against KCN intoxication were established for 10-12% and 5-6% methemoglobin levels induced by WR 6026. The effect of increased (over)

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19. Blood methemoglobin levels and intravenous sodium thiosulfate vs. cyanide intoxication was validated. The kinetics of methemoglobinemia induced by intramuscular injection of hydroxylamine hydrochloride was investigated. (AW). A protective index against KCN intoxication was established for a 10-12% blood methemoglobin level induced by prophylactic intramuscular injection of hydroxylamine hydrochloride and the optimum time for administration of hydroxylamine as a cyanide antidote was evaluated. Studies were also conducted to determine sodium nitrite's antidotal protection against cyanide intoxication in order to provide a data base so that sodium nitrite can be used as the standard for comparison of other methemoglobin-forming compounds. Pyridoxine hydrochloride (vitamin B6), pyridoxal 5-phosphate and α-ketoglutarate, compounds which do not induce methemoglobinemia, were also evaluated for their potential efficacy against lethal cyanide intoxication. Finally, the kinetics of methemoglobin formation were evaluated following oral administration of WR 242,511, another 8-aminoquinoline derivative. Results of the above studies showed that both WR 6026 and hydroxylamine hydrochloride are potent methemoglobin inducers which are capable of protecting against cyanide intoxication. Both antidotes can be administered in a form (orally or intramuscular injection) which would be practical in a battlefield situation. Both compounds must be administered prophylactically in order to be effective. Further studies are required to more fully evaluate the toxicity of these compounds prior to human use.

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SUMMARY

The purpose of this research was to measure in beagle dogs the degree of protection from lethal intravenous doses of potassium cyanide achieved by varying levels of methemoglobinemia in an attempt to identify one or more drugs which could be used for protection against cyanide intoxication in a battlefield situation where exposure to potentially lethal doses of cyanide is likely. This research program has been ongoing for three years. In the first year, the LD50 of intravenously administered potassium cyanide was determined to be 1.85 mg/kg body weight with a 95% confidence interval of 1.70 to 2.01 mg/kg. In addition, the chemical method for determining methemoglobin in the blood of dogs was validated and the clearance rate of increased levels of blood methemoglobin induced by WR 6026, an 8-aminoquinoline experimental anti-leishmanial drug, was determined.

During the second year, the effect of pre-existing levels of methemoglobin induced by WR 6026 and other drugs, primarily hydroxylamine hydrochloride, on the ability of the dog to resist cyanide intoxication was evaluated using the above obtained information. A "protective index" of 3.5 was established for a 10-12% blood methemoglobin level induced by WR 6026 administration, while a "protective index" of 1.75 was established for 5-6% methemoglobinemia induced by WR 6026 administration. A "protective index" of 3.75 was established for a 10-12% blood methemoglobin level induced by intramuscular injection of hydroxylamine hydrochloride.

The optimum time for therapeutic or prophylactic intramuscular injection of hydroxylamine hydrochloride as an antidote against cyanide intoxication was evaluated during the third year. Hydroxylamine hydrochloride appeared to be ineffective as a therapeutic agent against KCN intoxication. In contrast, hydroxylamine hydrochloride could be administered intramuscularly as short as one minute prior to KCN challenge and protect against lethal cyanide intoxication of up to at least 1.5xLD50.

Studies were also conducted during the third year to determine sodium nitrite's antidotal protection against cyanide intoxication in order to provide a data base so that sodium nitrite could be used as the standard for comparison of other methemoglobin-inducing compounds. Results of these studies indicated that $NaNO_2$ is an effective antidote against lethal cyanide intoxication when administered intravenously at a level of as low as 5 mg/kg either ten minutes prior to (prophylactic treatment) or immediately after (therapeutic treatment) cyanide injection. Sodium nitrite is also an effective antidote against cyanide intoxication when administered intramuscularly at a level as low as 5 mg/kg approximately ten minutes prior to KCN injection (prophylactic treatment). However, intramuscular administration of $NaNO_2$ did not protect against cyanide intoxication when given therapeutically, at least up to 20 mg/kg.

Pyridoxine hydrochloride (vitamin B6), pyridoxal 5-phosphate and α-ketoglutarate, compounds which do not induce methemoglobinemia, were also evaluated for their potential efficacy against lethal cyanide intoxication when administered prophylactically via different routes of administration. Pyridoxine hydrochloride and its physiologically active form, pyridoxal 5-phosphate, were ineffective as prophyloctic antidotes against KCN intoxication following intravenous administration. Intramuscular, prophylactic administration of α-ketoglutarate was also ineffective in preventing against lethal cyanide intoxication; however, intravenous prophylactic α-ketoglutarate administration was effective in preventing

against lethal cyanide intoxication. Oral administration of α-ketoglutarate was not effective as an antidote due to vomiting of the test solution.

Finally, the kinetics of methemoglobin formation and any related toxicity were evaluated following oral administration of WR 242,511, an 8-aminoquinoline derivative. Oral administration resulted in increased blcod methemoglobin levels, the magnitude of the increase being related to both the dose level and frequency of administration. Toxicity related to WR 242,511 administration included decreased activity, anorexia, decreased body weight, increased liver enzyme activity and death.

In conclusion, both WR 6026 and hydroxylamine hydrochloride are potent methemoglobin inducers which are capable of protecting against cyanide intoxication. Both antidotes can be administered in a form, orally or via intramuscular injection, which would be practical in a battlefield situation. Both compounds must be administered prophylactically, i.e., prior to cyanide exposure, in order to be effective. Further studies are required to more fully evaluate the toxicity of these compounds prior to human use.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in the report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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INTRODUCTION

Statement of the Problem

The exposure to lethal levels of cyanide gas has occurred in battlefield situations throughout the world during the past century and the threat of exposure to potentially lethal levels of cyanide remains a likely possibility in the future. Studies were conducted during the last three years evaluating the efficacy of selected compounds as to potential antidotes against cyanide intoxiciation. Results will be used in an attempt to develop a drug or regimen of drugs which can be used for protection against cyanide intoxication in a battlefield situation where the liklihood of the individual soldier being exposed to potentially lethal levels of cyanide exists. In the majority of studies, protection against lethal doses of potassium cyanide was achieved by increasing blood methemoglobin levels; however, other chemical agents were tested for potential efficacy against lethal cyanide intoxication in which the mechanism of protection did not involve an increase in blood methemoglobin levels. The beagle dog was used as the animal model in all experiments.

Background

Inorganic cyanides are poisonous by nature of the cytotoxic hypoxia they produce. Cyanide is a strong metabolic inhibitor, arresting cellular respiration by inactivating metallo-enzymes fundamental in the respiratory process.

Cyanide has a very high affinity for iron in the ferric state. When absorbed, it reacts readily with the trivalent iron of cytochrome oxidase in mitrochondria; cellular respiration is thus inhibited and cytotoxic hypoxia results. Since utilization of oxygen is blocked, venous blood is oxygenated and is almost as bright red as arterial blood. Respiration is stimulated because chemoreceptive cells respond as they do to decreased oxygen. A transient stage of CNS stimulation with hyperpnea and headache is observed; finally there are hypoxic convulsions and death due to respiratory arrest.

Treatment of cyanide poisoning must be rapid to be effective. Since toxicity results from binding to the ferric form of cytochrome oxidase, treatment is aimed at prevention or reversal of such binding by providing a large pool of ferric iron to compete for cyanide. An effective mechanism is to administer substances, such as sodium nitrite, inorganic hydroxylamine, aminophenols, p-aminopropiophenone or 8-aminoquinoline, that oxidize hemoglobin to methemoglobin. Methemoglobin competes with cytochrome oxidase for the cyanide ion; the reaction favors methemoglobin because of mass action. Cyanmethemoglobin is formed, and cytochrome oxidase is restored.

The major mechanism for removing cyanide from the body is its enzymatic conversion, by the mitochondrial enzyme rhodanese (transsulfurase), to thiocyanate, which is relatively nontoxic. To accelerate detoxication, thiosulfate can be administered intraveously (50 ml of a 25% aqueous solution), and the thiocyanate formed is readily excreted in the urine. Way and associates[1] demonstrated that nitrite increased the LD50 of potassium cyanide in mice from 11 mg/kg to 21 mg/kg; administration of thiosulfate increases the value to 35 mg/kg, while with nitrite followed by thiosulfate the LD50 is 52 mg/kg. Chen and Rose[2] reported that 48 of 49 cases of acute poisoning by cyanide in man were treated successfully with such therapy. Marrs et. al.[3] found that five of six dogs survived an intravenous injection

of approximately 2 LD50's of hydrogen cyanide given when methemoglobin levels (induced by 4-dimethylaminophenol) reached 8 to 10%.

Oxygen alone, even at hyperbaric pressures, has only a slight protective effect in cyanide poisoning; however, it dramatically potentiates the protective effects of thiosulfate or of nitrite and thiosulfate[1,4,5]. The mechanism for this action is not clear, but the intracellular oxygen tension may be high enough to cause nonenzymatic oxidation of reduced cytochromes or oxygen may displace cyanide from cytochrome oxidase by mass action.

Part 1

Summary of Previous Work

The purpose of this research was to measure in Beagle dogs the degree of protection from lethal intravenous doses of potassium cyanide achieved by varying levels of methemoglobinemia in order to identify one or more drugs which can be used for protection against cyanide intoxication in a battlefield situation where exposure to potentially lethal doses of cyanide is likely.

Initially, the acute LD50 of a single dose of a potassium cyanide (KCN) solution was determined in male and female Beagle dogs when administered intravenously over a one minute period. The calculated acute intravenous LD50 of the KCN solution in male and female animals combined was found to be 1.85 mg/kg body weight with a 95% confidence interval of 1.70 to 2.01 mg/kg.

Secondly, the procedure for the determination of methemoglobin[6] was evaluated for recovery, linearity and reproducibility.

The recovery of known amounts of methemoglobin in dog plasma showed a consistent % recovery from standards of 2.5 to 20.0 g/dl, resulting in a mean % recovery of methemoglobin of 98.7 $\pm$ 2.83% with a coefficient of variation of 2.86%. Lower recovery occurred in standards less than 2.5 g/dl, where the 1.25 and 0.63 g/dl standards resulted in 95% and 93% recovery, respectively.

The linearity of methemoglobin determinations using known amounts of canine methemoglobin dissolved in pooled dog plasma yielded a correlation coefficient of 0.9996, slope of 0.0712 and intercept of 0.036.

The reproducibility of methemoglobin measurement was conducted in vitro and in vivo by inducing methemoglobinemia in the dog or in dog whole blood samples by the addition of standard concentrations of sodium nitrite. In vitro experiments were performed on two separate blood samples, resulting in a mean coefficient of variation of 2.35% and 1.52%. In vivo reproducibility of methemoglobin measurement was performed on two dogs over a period of 2 hours. A mean coefficient of variation of 1.90% and 1.33% was achieved.

In conclusion, the data compiled using the Tietz procedure for the determination of methemoglobin indicated that it was an accurate, linear and reproducible method for determination of higher than normal levels of methemoglobin.

Subsequently, blood methemoglobin levels were determined following administration of WR 6026, an 8-aminoquinoline experimental anti-leishmanial drug. Eight dogs were divided into two groups (2 per sex per group) and administered WR 6026 either as a salt via a capsule (Group A) or as an aqueous solution via gastric intubation (Group B) at a dose of 4.83 mg/kg body weight. Each dog was dosed once daily for four consecutive days. Blood methemoglobin levels were determined two and one day prior to administration and daily for 12 days after administration of the fourth dose (methemoglobin levels were not determined during the 4 day dosing period).

Group mean methemoglobin values attained their highest levels one day after the last dose (day 5) for both dosage forms of WR 6026. The average peak level of methemoglobin was approximately 20% when WR 6026 was administered via capsule and approximately 19% when administered as a solution. For both dosage forms, mean methemoglobin values decreased to a level of approximately 10% five days after the last dose (day 9). No appreciable differences in mean methemoglobin levels at any time period were apparent between the salt and solution forms of WR 6026.

The effect of pre-existing levels of blood methemoglobin (10-12%) induced by WR 6026 on the ability of the Beagle dog to resist lethal cyanide (KCN)

intoxication was investigated next. Five groups of five dogs each were administered WR 6026 once daily for at least four consecutive days and then given an intravenous injection of KCN at a level of 1xLD50, 2xLD50, 3xLD50, 3.5xLD50 or 4xLD50 when the blood methemoglobin level had decreased to 10-12%. The number of surviving animals following cyanide administration in each group was then compared to the LD50 mortality curve for cyanide determined in animals not previously administered WR 6026 in an attempt to establish a "protective index" for a 10-12% methemoglobin level. All animals injected with cyanide at 1xLD50, 2xLD50 or 3xLD50 survived the challenge, while the five animals injected with cyanide at 4xLD50 died following the injection. For four animals injected with cyanide at 3.5xLD50, two died and two survived. Analysis of the data showed an ED50 (dose at which 50% of the animals showed an effect) value of 6.48 mg/kg. This value was 3.5 times greater than the LD50 value (1.85 mg/kg) obtained with a single injection of KCN with non-induced ("normal") methemoglobin levels. Thus, a "protective index" of 3.5 was established for a 10-12% blood methemoglobin level induced by WR 6026 administration.

In the next experiment, the effect of pre-existing blood methemoglobin levels of 5-6% induced by WR 6026 on the ability to resist cyanide intoxication was determined. Three groups of five dogs each were administered WR 6026 once daily for three consecutive days and then given an injection of potassium cyanide at a level of 1xLD50, 2xLD50 or 2.5xLD50 when the blood methemoglobin level had decreased to approximately 5-6%. The number of surviving animals following cyanide administration in each test group was then compared to the LD50 mortality curve for cyanide determined in animals not previously administered WR 6026 in an attempt to establish a "protective index" for a 5-6% methemoglobin level. All animals injected with cyanide at the LD50 level survived the injection. Two animals injected with cyanide at a level of 2xLD50 died following the injection, while the other three animals injected at this level survived the challenge injection. All five dogs injected with cyanide at a level of 2.5xLD50 died following injection. Analysis of the data showed an ED50 value of 3.23 mg/kg. This value was 1.75 times greater than the LD50 value obtained with a single injection of KCN in dogs with noninduced ("normal") blood methemoglobin levels. Thus, a "protective index" of 1.75 was established for a 5-6% blood methemoglobin level induced by WR 6026 administration.

The effect of a single dose of WR 6026 on blood methemoglobin levels over a 72 hour period and the relationship, if any, between blood glucose levels and blood methemoglobin levels induced by WR 6026 administration were determined in the next study. Six male animals were equally divided into two test groups (A and B) and a control group (C). Each dog in group A received a single dose, via capsule, of WR 6026 at a level of 9.66 mg/kg body weight, while each dog in group B received a similar dose of WR 6026 at a level of 14.49 mg/kg body weight. Animals in group C received no WR 6026. Blood methemoglobin levels for each dog in groups A and B were determined one day prior to administration of WR 6026 (day -1), immediately prior to dosing, and .25 (15 min.), .50 (30 min.), 1, 2, 4, 6, 8, 24, 48 and 72 hours after dosing. Hemoglobin, hematocrit, and serum glucose levels were determined for each dog in groups A and B immediately prior to administration of WR 6026 and 4, 8, 24, 48 and 72 hours after dosing. For the control dogs, glucose levels only were determined at the same time intervals as for the dogs in groups A and B. One dog in each group (test and control) was provided food for the normal two hour

period each day of the study while the other dog from each group (test and control) was fed ad libitum during the course of the study.

Results of this study indicated that the interval between administration of WR 6026 and feeding, and ultimate blood glucose levels, may have had an effect on blood methemoglobin levels. The effect appeared to be an inverse one in that the higher the glucose levels, the lower the methemoglobin levels. It was recommended that an additional study be conducted to determine the effects of feeding and blood glucose levels on the induction of methemoglobinemia following WR 6026 administration.

It was also apparent from the results of this study that the dog can be administered a single dose of up to approximately 14.5 mg/kg body weight of WR 6026 without showing any signs of drug-related toxicity. Furthermore, blood methemoglobin levels of up to nearly 10% can be induced with a single administration of WR 6026, which may be useful in reducing the amount of time needed to conduct further studies with WR 6026.

The effect of feeding on blood methemoglobin levels induced by WR 6026 was determined next. Dogs were divided into two groups, each consisting of one male and two females. Each dog in both groups was administered WR 6026 once daily, via capsule, at a level of 4.83 mg/kg for four consecutive days. All three dogs in group A were fed approximately one-half hour after WR 6026 administration, while dogs in group B were fed approximately four hours after WR 6026 administration. Blood methemoglobin levels for each dog in both groups were determined one day prior to administration of WR 6026 (day -1), daily during the dosing period (predose) and 1 day after the last dose of WR 6026.

Results of the methemoglobin determinations on day 5 showed essentially no differences in individual methemoglobin levels between groups. Group mean % methemoglobin levels on day 5 were similar (A = 12.5%, B = 11.1%). Thus, results indicated that the interval between administration of WR 6026 and feeding (at least up to 4 hours) did not appear to have an effect on blood methemoglobin levels.

The kinetics of methemoglobinemia induced by intramuscular (i.m.) injection of hydroxylamine hydrochloride was determined in the next study. Two dogs (1M,1F) received an i.m. injection of hydroxylamine hydrochloride in the thigh muscle of the left hind leg at a level of 5 mg/kg body weight, while two dogs (1M,1F) received a similar injection of hydroxylamine hydrochloride at a level of 10 mg/kg body weight. A 5% hydroxylamine hydrochloride solution was prepared in sterile saline and injected at a level of 0.1 ml/kg for the 5 mg/kg dogs and 0.2 ml/kg for the 10 mg/kg dogs. Blood was collected from each dog and methemoglobin levels determined immediately prior to injection and at 5, 15, 30, 60, 120, 240 and 360 minutes after injection. For both animals at the 5 mg/kg level, peak methemoglobin levels were present 15 minutes after injection, while peak methemoglobin levels were present within 5 minutes after injection for both animals at the 10 mg/kg level. Thus, results indicated that intramuscular injection of hydroxylamine hydrochloride caused an increase in blood methemoglobin levels immediately after administration. Since methemoglobin levels of 10-12% were induced within 5 minutes after injection of hydroxylamine hydrochloride at the 10 mg/kg dose level, it appeared that this compound could be used at this concentration in further studies with potassium cyanide. An additional methemoglobinemia kinetic study with hydroxylamine hydrochloride (intramuscular injection) at a concentration of 17.5% and dose level of 10 mg/kg resulted in methemoglobin levels of 10-12%

within 5-10 minutes post injection and peak methemoglobin levels (13-14%) 15 minutes after injection.

The effect of increased blood methemoglobin levels and intravenous sodium thiosulfate ($Na_2S_2O_3$) administration versus potassium cyanide (KCN) intoxication was investigated next. Three female dogs were administered WR 6026 once daily, via capsule, at a level of 4.83 mg/kg for four consecutive days. Blood methemoglobin levels for each dog were determined one day prior to administration of WR 6026, one day after the last dose of WR 6026 (day 5), and then daily until administration of KCN. A single intravenous injection of KCN (3.5xLD50) was administered to each dog when the blood methemoglobin level had decreased to approximately 10-12%. Immediately following KCN injection, each animal was administered a single intravenous injection of sodium thiosulfate solution (150 mg/ml) at a level of 150 mg/kg. The $Na_2S_2S_3$ solution was administered as a bolus over a period of three minutes.

Immediately following KCN injection, all dogs exhibited tonic contractions and loss of the corneal reflex. Two animals showed excessive salivation, while one exhibited decreased respiration and one a complete loss of respiration. Following $Na_2S_2O_3$ administration, an immediate alleviation of these symptoms, characterized by a subsiding of the tonic contructions, return of the corneal reflex and return of normal respiration, was noted in all dogs. All dogs appeared completely normal within 15 to 60 minutes after KCN injection.

In conclusion, intravenous administration of sodium thiosulfate under the conditions of this experiment resulted in noticeable alleviation of toxic symptoms and an increase in the expected survival rate, in relation to previous studies conducted under similar circumstances with cyanide followed by intramuscular injection of sodium thiosulfate, in which no alleviation of toxic symptoms was present.

In another experiment, the prophylactic and therapeutic effect of intramuscular hydroxylamine hydrochloride administration versus potassium cyanide (KCN) intoxication was determined. For the prophylactic phase, one male dog received an intramuscular injection of a 5% solution (in 0.9% sterile saline) of hydroxylamine hydrochloride in the thigh muscle of the left hind leg at a level of 10 mg/kg (0.2 ml/kg). Approximately ten minutes after hydroxylamine injection, the dog was administered a single intravenous injection of KCN at a level of 2xLD50 (3.70 mg/kg). In addition, the blood methemoglobin level in this dog was determined immediately prior to KCN administration. For the therapeutic phase, one male dog was administered a single intravenous injection of KCN at a level of 2xLD50 (3.70 mg/kg) followed by an intramuscular injection of a 5% solution of hydroxylamine hydrochloride at a level of 10 mg/kg (0.2 ml/kg).

For the animal which received the prophylactic dose of hydroxylamine hydrochloride prior to KCN administration, the only noticeable effect following KCN injection was a slight increase in respiration; however, the animal appeared normal within five minutes after injection. Analysis of the blood taken from this animal prior to KCN injection showed a methemoglobin level of 9.47%. For the animal which received the therapeutic dose of hydroxylamine hydrochloride after KCN injection, no alleviation of the KCN-induced signs of toxicity (tonic contractions, loss of corneal reflex) was apparent. The animal died within ten minutes after KCN administration.

Thus, results indicated that hydroxylamine hydrochloride had a prophylactic, but not therapeutic, effect against lethal KCN intoxication when

administered intramuscularly at a level of 10 mg/kg under the conditions of this study.

The effect of pre-existing levels of blood methemoglobin (10-12%) induced by prophylactic administration of hydroxylamine hydrochloride on the ability of the Beagle dog to resist lethal cyanide (KCN) intoxication was determined next. Three groups of five dogs each received an intramuscular injection of hydroxylamine hydrochloride (175 mg/ml) at a level of 10 mg/kg body weight. Approximately ten minutes after hydroxylamine administration, each dog was given an intravenous injection of KCN at a level of 3xLD50, 3.5xLD50 or 4xLD50. The number of surviving animals following cyanide administration in each group was then compared to the LD50 mortality curve for cyanide determined in animals not previously exposed to hydroxylamine hydrochloride in an attempt to establish a "protective index" for a 10-12% methemoglobin level induced by i.m. injection of hydroxylamine hydrochloride. Analysis of the mortality data at the completion of dosing showed an ED50 value of 6.94 mg/kg. This value was 3.75 times greater than the LD50 value (1.85 mg/kg) obtained with a single injection of KCN with non-induced (normal) methemoglobin levels. Thus a "protective index" of 3.75 was established for a 10-12% blood methemoglobin level induced by intramuscular injection of hydroxylamine hydrochloride.

The optimum time for prophylactic and therapeutic intramuscular injection of hydroxylamine hydrochloride as an antidote against lethal cyanide (KCN) intoxication was then determined. Twelve dogs in group A and four dogs in group B received a lethal injection of KCN at a level of 1.5xLD50. The KCN solution was administered over a period of 15, 20, 30 or 60 seconds. Immediately (group A) or one minute (group B) after KCN administration, each animal received a single intramuscular injection of a 17.5% solution of hydroxylamine hydrochloride at a dose of 10 mg/kg into the caudal thigh muscle of the left hind leg. Five animals in group C received a single intramuscular injection of hydroxylamine hydrochloride followed by a lethal injection of KCN (1.5xLD50) one minute <u>after</u> hydroxylamine administration.

Administration of hydroxylamine hydrochloride immediately after KCN injection resulted in the survival of 6 of 12 animals. However, survival of these animals appeared to be related to the rate of cyanide injection rather than to the hydroxylamine injection, i.e., the faster the cyanide can be injected, the shorter the interval between initial effects induced by KCN and alleviation of these effects by injection of hydroxylamine. No alleviation of toxic symptoms, decreased severity of the toxic response or decreased time of recovery from toxic symptoms was observed in any animal administered hydroxylamine immediately after KCN injection. All animals which received hydroxylamine hydrochloride one minute after KCN administration died following injection. All animals which received hydroxylamine hydrochloride one minute prior to KCN injection survived the cyanide challenge.

In conclusion, hydroxylamine hydrochloride appeared to be ineffective as a therapeutic agent against KCN intoxication. There appeared to be no optimum time for therapeutic intramuscular administration of hydroxylamine as an antidote against KCN intoxication. In contrast, intramuscular injection of hydroxylamine hydrochloride appeared to be effective as a prophylactic agent against KCN intoxication. Hydroxylamine hydrochloride could be administered intramuscularly as short as one minute prior to KCN challenge and protect against lethal cyanide intoxication of up to at least 1.5xLD50.

Finally, studies were conducted to determine sodium nitrite's antidotal protection against cyanide intoxication in order to generate data so that sodium nitrite could be used as the standard for comparison of other methemoglobin-forming compounds. Protection was determined for both prophylactic and therapeutic administration and for both intravenous and intramuscular injection. Groups of 3 dogs each received either an intravenous or intramuscular injection of 5, 10 or 20 mg/kg $NaNO_2$ (10% aqueous solution) either ten minutes before (prophylactic experiment) or immediately after (therapeutic experiment) cyanide administration. Cyanide was administered intravenously at a level of 3.70 mg/kg (2xLD50).

For the animals in which $NaNO_2$ was administered intravenously, all three animals treated prophylactically survived at the 5 mg/kg level (only level tested) while all three animals treated therapeutically survived at the 5 and 20 mg/kg levels and two of three animals treated therapeutically survived at the 10 mg/kg level. For the animals in which $NaNO_2$ was administered intramuscularly, all three animals treated prophylactically survived at the 10 and 20 mg/kg levels while two of three animals treated prophylactically survived at the 5 mg/kg level. All three animals treated therapeutically died at the 20 mg/kg level (only level tested).

Thus, results of these studies indicated that $NaNO_2$ is an effective antidote against lethal cyanide intoxication when administered intravenously at a level of as low as 5 mg/kg either ten minutes prior to (prophylactic treatment) or immediately after (therapeutic treatment) cyanide injection. Sodium nitrite is also an effective antidote against cyanide intoxication when administered intramuscularly at a level as low as 5 mg/kg approximately ten minutes prior to KCN injection (prophylactic treatment). However, intramuscular administration of $NaNO_2$ did not protect against cyanide intoxication when given therapeutically, at least up to 20 mg/kg.

Part 2

Evaluation of the Prophylactic Effect of Pyridoxine Hydrochloride and Pyridoxal 5-Phosphate Against Potassium Cyanide Intoxication Following Intravenous Injection (Pilot Study)

INTRODUCTION

The purpose of this pilot study was to determine the effect of prophylactic intravenous administration of pyridoxine hydrochloride (vitamin B6; PN) and its active cofactor, pyridoxal 5-phosphate (PLP), when used as an antidote against lethal potassium cyanide (KCN) intoxication in the dog. The study was conducted from September 12, 1986 to September 30, 1986. All raw data and pertinent records are stored in the FDRL archives and are available upon request.

MATERIALS AND METHODS

Test Articles

Pyridoxine monohydrochloride (vitamin B6; catalog no. P-9755) and pyridoxal 5-phosphate (catalog no. P-9255) were purchased from Sigma Chemical Co., St. Louis, MO and assigned FDRL identification numbers 86-0524 and 86-0525, respectively. Both compounds were stored dessicated and in the dark at 0°C. Potassium cyanide (certified A.C.S., catalog no. P350501; lot no. ERT) was purchased from Mallinckrodt, Inc., St. Louis, MO and assigned FDRL identification number 83-0377.

Animals and Animal Care

Animal husbandry conformed to the standards established in "Guide for the Care and Use of Laboratory Animals", DHEW Publication (NIH) No. 85-23.

Seven female purebred Beagle dogs, approximately 9 to 12 months of age and weighing approximately 7.0-10.0 kg were used in these experiments. Animals were individually housed in pens with hardwood chip bedding ("Beta Chips", Northeastern Products Corporation, Warrensburg, NY 12885) and maintained in an environment-controlled room (temperature 64-72°F; relative humidity 40-70%) with a 12 hour light-dark cycle. Animals were acclimated to the environment for a minimum of 2 weeks prior to initiation of the experiment. During this period, animals were observed daily for any clinical signs of disease or other abnormalities which would eliminate them from the study. Animals received daily portions of approximately 400 g/dog of Big Red Nuggets dog food (Agway, Syracuse, NY) and tap water ad libitum. Food was presented for approximately 2 hours daily.

Animals were divided into three groups and administered PN, PLP and KCN as follows:

Group	No. of Animals	Level of Pyridoxine (mg/kg)	Level of Pyridoxal 5-Phosphate (mg/kg)	Level of Potassium Cyanide (mg/kg)
A	3	300	-	3.70[a]
B	3	-	300[b]	3.70
C	1	-	300[c]	3.70

[a] 2xLD50.
[b] Saline vehicle.
[c] Phosphate buffer vehicle.

Each animal was identified by an ear tattoo number and a cage card containing the project number and the ear tattoo number.

Experimental Procedures

Dose Preparation and Administration: PN and PLP solutions were prepared immediately prior to administration and administered via the cephalic vein (intravenous injection) at a level of 300 mg/kg body weight. Both solutions were prepared at a concentration of 300 mg/ml (30%) and were administered at a volume of 1.0 ml/kg. Pyridoxine hydrochloride was prepared in sterile water for injection. The pH of the solution was 3.0. The pyridoxal 5-phosphate solution used to dose the animals in group B was prepared in physiological (0.85% NaCl) saline. The pH of this solution was adjusted to 7.0 with 5N NaOH prior to dosing. The PLP solution used to dose the group C animal was prepared in 1M phosphate buffer (pH 8). The pH of this solution was adjusted to 7.4 with 1N NaOH prior to injection.

A lethal dose of KCN (2xLD50) was administered to each dog approximately ten minutes after PN or PLP injection. The potassium cyanide solution was prepared by dissolving an appropriate amount of KCN in an appropriate volume of physiological (0.85% NaCl) saline. The concentration of the KCN solution was checked prior to dosing by use of a titrimetric method[7]. Analyses showed that solutions were mixed to within $\pm$ 0.05 mg/ml of the theoretical value. The KCN was administered at a constant volume (1.0 ml/kg body weight). Each animal was suspended in a sling and the cephalic vein of one forelimb was cannulated using a L-CATH™ IV Cath Placement Set (catheter - 23GA, 2.4 in; needle - 20GA; Luther Medical Products, Costa Mesa, CA, 92626; catalog No. 23P6). The KCN solution was administered intravenously via the cephalic vein over a period of 60 seconds.

Observations: Animals were weighed prior to administration of PN or PLP. Individual body weights are given in Table 2-4. Animals were observed for mortality and toxic signs frequently after dosing.

Clinical Laboratory Studies: One (day -1) or three (day -3) days prior to initiation of the dosing, hemoglobin and hematocrit levels were determined and a white blood cell count conducted on all animals. Any animal whose values for all these parameters fell below or exceeded the range of FDRL historical values taken from control Beagle dogs of similar age for the same parameters (WBC count: 6,000-14,800/µl for males, 7,900-16,500/µl for females; Hgb: 13.7-18.9 gm% for males, 14.3-18.3 gm% for females; Hct: 40-56% for males, 42-54% for females) were disqualified from the study and replaced by another animal whose values were within the specificed ranges. The clinical methods used are referenced in Appendix I.

Blood methemoglobin levels for each dog in groups A and B were determined one or three days prior to dosing, immediately prior to dosing, and then 5, 10, 15 and 30 minutes after PN or PLP administration, when possible, according to the procedure of Tietz[6]. Hemoglobin and hematocrit levels were determined for all animals at the same time methemoglobin values were detemined. Methemoglobin values were not determined for the one group C animal.

RESULTS AND DISCUSSION

Methemoglobin Levels, Mortality and Observations

A summary of dosing (KCN, PN, PLP) and mortality data is given in Table 2-1, while individual methemoglobin levels prior to (baseline) and following dosing are given in Table 2-2. Observations are summarized in Table 2-3.

All three animals administered pyridoxine hydrochloride, all three animals administered pyridoxal 5-phosphate in water, and the one animal injected with pyridoxal 5-phosphate in phosphate buffer died within 3-12 minutes after KCN injection. Blood methemoglobin levels were not increased following administration of PN or PLP.

Injection of PN did not appear to cause any outward signs of toxicity. However, injection of PLP in water resulted in several signs of toxicity, including increased respiration, decreased activity, dry heaves, and yellow-colored sclera and gums, in 2 or 3 dogs approximately two minutes after injection. Dry heaves and yellow gums and sclera were also present in the dog injected with PLP in phosphate buffer. The yellow color in the sclera of the eyes and in the gums matched the yellowish color of the PLP solutions. Immediately after KCN administration and prior to death, all animals exhibited tonic contractions, loss of the corneal reflex and loss of respiration.

Hematology

Individual hematology data (white blood cell count, hemoglobin and hematocrit levels) obtained during the study are given in Appendix II. No significant decreases in hemoglobin or hematocrit levels were noted after PN or PLP administration.

CONCLUSION

Pyridoxine hydrochloride and its physiologically active form, pyridoxal 5-phosphate, appear to be ineffective as prophylactic antidotes against KCN intoxication when administered under the conditions of this experiment.

Table 2-1

Summary of Dosing and Mortality Data

Group and Treatment	Animal and Sex	Level of PN or PLP (mg/kg BW)	Level of KCN (mg/kg BW)	Fate
A	4AVX4 F	300	3.70[a]	D[b]
Pyridoxine	5AWJ4 F	300	3.70	D
Hydrochloride	5AVN7 F	300	3.70	D
B	5AVK5 F	300	3.70	D
Pyridoxal	5AWG2 F	300	3.70	D
5-Phosphate[c]	5AWI3 F	300	3.70	D
C	5AVX3 F	300	3.70	D
Pyridoxal 5-Phosphate[d]				

[a] 3.70 = 2xLD50.

[b] D = Died.

[c] Saline vehicle.

[d] Phosphate buffer vehicle.

Table 2-2

Individual Methemoglobin Levels Following Administration of Pyridoxine Hydrochloride and Pyridoxal 5-Phosphate

Group and Treatment	Animal and Sex	Dosage Level (mg/kg BW)	% Methemoglobin -Time Period[a]- Pretest	Predose	5 Min.	10 Min.	15 Min.	30 Min.
A	4AVX4 F	300	1.47	1.17	1.06	0.63	0.44	-
Pyridoxine	5AWJ4 F	300	0.84	1.14	0.54	0.38	-	-
Hydrochloride	5AVN7 F	300	0.60	0.97	-	-	-	-
B	5AVK5 F	300	1.09	1.06	-	-	-	-
Pyridoxal	5AWG2 F	300	0.94	0.96	0.78	0.94	-	-
5-Phosphate	5AWI3 F	300	0.78	0.88	0.55	0.60	-	0.62

[a] Baseline values were determined on day -3 (group A) or day -1 (group B) and prior to dosing; animals were administered pyridoxine hydrochloride or pyridoxal 5-phosphate approximately ten minutes prior to KCN administration; blood methemoglobin levels were determined approximately 5, 10, 15 and 30 minutes after PN or PLP administration when possible.

Table 2-3

Summary of Observations

Observation	Treatment		
	Pyridoxine Hydrochloride	Pyridoxal 5-Phosphate[a]	Pyridoxal 5-Phosphate[b]
tonic contractions	3/3[c]	3/3	1/1
loss of corneal reflex	3/3	3/3	1/1
loss of respiration	3/3	3/3	1/1
increased respiration	0/3	2/3	0/1
decreased activity	0/3	2/3	0/1
dry heaves	0/3	3/3	1/1
yellow sclera and gums	0/3	3/3	1/1

[a] Saline vehicle.

[b] Phosphate buffer vehicle.

[c] Number of animals with observation/Number of animals dosed.

Table 2-4

Individual Body Weight Data

Group and Treatment	Animal and Sex	Level of PN or PLP (mg/kg BW)	Body Weight (kg) at Dosing
A	4AVX4 F	300	8.2
Pyridoxine	5AWJ4 F	300	9.4
Hydrochloride	5AVN7 F	300	7.0
B	5AVK5 F	300	7.4
Pyridoxal 5-Phosphate[a]	5AWG2 F	300	8.1
	5AWI3 F	300	8.5
C	5AVX3 F	300	9.5
Pyridoxal 5-Phosphate[b]			

[a] Saline vehicle.

[b] Phosphate buffer vehicle.

Part 3

Evaluation of the Prophylactic Effect of α-Ketoglutarate Against Potassium Cyanide Intoxication Following Intravenous Injection, Intramuscular Injection and Oral Administration

PHASE 1: Evaluation of the Prophylactic Effect of Intramuscular and Intravenous Injection of α-Ketoglutarate Against Potassium Cyanide Intoxication

A pilot study was conducted to evaluate the prophylactic effect of intramuscular and intravenous administration of α-ketoglutarate (α-KG) against lethal cyanide intoxication in the Beagle dog. One male dog received an intramuscular injection of α-KG (500 mg/ml aqueous solution) at a dose of 0.5 g/kg body weight. The dose was delivered in four intramuscular injections (3 ml/injection site) into the thigh muscle of the left hind leg (2 injections in each thigh). One female dog received a single intravenous injection (via cephalic vein) of α-KG (500 mg/ml aqueous solution) at a dose of 1.0 g/kg body weight. Approximately ten minutes after α-KG administration, each dog was given an intravenous injection (via jugular vein) of KCN at a level of 3.70 mg/kg (2xLD50). Blood methemoglobin levels for each dog were determined immediately prior to α-KG administration, and then 2, 5, 10, 15, 30 and 60 minutes after α-KG administration, if possible. Two female rats received a single intraperitoneal injection of KCN at 2xLD50 (11 mg/kg) to serve as positive controls.

Individual methemoglobin levels prior to (baseline) and following administration of α-KG are given in Table 3-1. Injection of α-KG had no effect on blood methemoglobin levels in either animal. Values were considered "baseline" at all times after α-KG administration. The animal in which α-KG was administered intramuscularly died 8 minutes after KCN injection, while the dog which received the intravenous injection of α-KG survived the cyanide challenge injection. Both dogs exhibited tonic contractions, loss of the corneal reflex and loss of respiration immediately after KCN injection. Three minutes after KCN injection a return of respiration was observed in the surviving animal, followed by a return of the corneal reflex (6 minutes post-injection) and then increased respiration (11 minutes post-injection). The surviving animal still showed decreased activity 2 hours after KCN injection, however it appeared normal by 24 hours post-injection. Both rats died 3 minutes after KCN administration.

In conclusion, it appears that intramuscular, prophylactic administration of α-KG at the concentration and dose level used in this study is ineffective in preventing against lethal cyanide intoxication. Conversely, intravenous prophylactic administration of α-KG at the concentration and dose level used in this study was effective in preventing against lethal cyanide intoxication.

Table 3-1

Individual Methemoglobin Levels Following Administration of α-Ketoglutarate

Animal No. and Sex	Dosage Level (g/kg BW)	Route of Administration	% Methemoglobin - Time Period[a]						
			Predose	2 Min.	5 Min.	10 Min.	15 Min.	30 Min.	60 Min.
5AWS1 M	0.5	Intramuscular	0.52	1.19	0.95	0.86	0.49	Died	-
5ABS3 F	1.0	Intravenous	0.61	0.70	0.58	0.75	-	1.02	0.88

[a] Baseline values were determined prior to dosing; animals were administered α-ketoglutarate ten minutes prior to KCN administration; blood methemoglobin levels were determined approximately 2, 5, 10, 15, 30 and 60 minutes after α-ketoglutarate administration when possible.

PHASE 2: Evaluation of the Prophylactic Effect of Oral Administration of α-Ketoglutarate Against Potassium Cyanide Intoxication

A pilot study was conducted to evaluate the prophylactic effect of oral administration of α-ketoglutarate against lethal cyanide intoxication in the Beagle dog. In the initial phase, two female dogs were administered α-ketoglutaric acid (800 mg/ml in 0.1 M phosphate buffer) via oral gavage at a dose of 4 g/kg body weight. The α-KG solution was administered at a level of 5.0 ml/kg, so that each dog received a total volume of 43 or 45 ml. The pH of the α-KG solution was approximately 2.

Approximately one minute after administration of α-KG each dog vomited the bolus of test solution. Each dog continued to vomit repeatedly during the hour after dosing. Fresh blood appeared in the vomitus approximately one-half hour after dosing. In addition to the vomiting, both dogs exhibited decreased activity after dosing. No cyanide was administered to either animal due to the vomiting of the test solution. Both dogs died within 24 hours after dosing. Necropsy examination of one of the two dogs showed severe hemorrhagic ulceration of the stomach due to irritation of the test solution.

Two additional female dogs were administered α-ketoglutaric acid (285.7 mg/ml in 0.1 M phosphate buffer) via oral gavage at a dose of 2 g/kg body weight. The α-KG solution was administered at a level of 7.0 ml/kg, so that each dog received a total volume of 64 or 66 ml. The pH of the α-KG solution was adjusted to pH 7.45 with 5N NaOH. One vehicle control animal was dosed (gavage) with 0.1 M phosphate buffer only.

Both dogs which received the α-KG solution vomited the bolus of test solution either three or ten minutes after dosing. No blood was present in the vomitus. Both dogs appeared normal after vomiting. The dog which received only the phosphate buffer did not vomit. No cyanide was administered to either dog which received the α-KG solution.

In conclusion, oral administration of α-ketoglutarate at the concentration and dose level used in this pilot study would not be effective as a possible antidote against lethal cyanide intoxication due to vomiting of the test solution. An acidic dosing solution caused gastric irritation with resulting hemorrhage and ulceration. Although a neutral dosing solution appeared not to cause any gastric irritation, it did result in vomiting.

Part 4

Evaluation of the Kinetics of Methemoglobin

Formation Induced by Oral Administration

of WR 242,511

INTRODUCTION

The purpose of this study was to evaluate the kinetics of methemoglobin formation by establishing the dose response relationship between administration of the test article (WR 242,511) and the rate of methemoglobin clearance from the blood. Any toxicity related to WR 242,511 administration was also evaluated. The study was performed from November 17, 1986 to December 19, 1986. All raw data and pertinent records are stored in the FDRL archives and are available upon request.

MATERIALS AND METHODS

Test Article

The test article identified as WR 242,511, BL 09417, 862961A1 was received from the Sponsor on October 24, 1986 and assigned FDRL identification number 86-0609. The compound was stored dessicated in the dark at an average of -22° to -24° C.

Animals and Animal Care

Animal husbandry conformed to the standards established in "Guide for the Care and Use of Laboratory Animals", DHEW Publication (NIH) No. 85-23. Six female purebred Beagle dogs, approximately 9 to 15 months of age and weighing 7.6 to 10.1 kg were used in this study. Animals were individually housed in pens with hardwood chip bedding ("Beta Chips", Northeastern Products Corporation, Warrensburg, NY 12885) and maintained in an environment-controlled room (temperature 64° to 72° F; relative humidity 40 to 70%) with a 12 hour light-dark cycle. Animals were acclimated to the environment for a minimum of 2 weeks prior to initiation of the experiment. During this period, the animals were observed daily for any clinical signs of disease or other abnormalities. Animals received daily portions (approximately 350 g/dog) of "Big Red Nuggets" dog food (Agway, Syracuse, New York) and tap water ad libitum. Food was offered for approximately four hours each day.

Animals (females) were divided into three groups and administered WR 242,511 as follows:

Group	No. of Animals	Level of WR 242,511 (mg/kg)	Frequency of Dosing
A	2	7.024	once daily for 4 consecutive days
B	2	7.024	single dose
C	2	14.048	single dose

Each animal was identified by an ear tattoo number and a cage card containing the project number, group, and the ear tattoo number.

Experimental Procedures

Dose Preparation and Administration: WR 242,511 was administered orally via gelatin capsule. The two females assigned to group "A" were given a single daily dose (7.024 mg/kg) for four (4) consecutive days (study days 1-4). Groups "B" and "C" (two females per group) were given a single dose on study day one at levels of 7.024 and 14.048 mg/kg, respectively.

Observations: Animals were weighed prior to WR 242,511 administration and then daily until termination (weekends excluded). Animals were observed for mortality and toxic signs frequently during the day of dosing and then daily until termination.

Clinical Laboratory Studies: Hemoglobin, hematocrit and methemoglobin levels were determined for group "A" animal(s) on days -1, 1 (prior to WR 242,511 administration), 2-5, 8-12, 15-19, 22-26, 29, 30 and 33. Blood methemoglobin levels were determined according to the procedure of Tietz[6]. In addition, serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase levels were determined for group "A" animals on days -1, 1 (prior to WR 242,511 administration), 2-5 (prior to dosing on days 2-4), 12, 19 and 26.

Hemoglobin, hematocrit and methemoglobin levels for each animal in groups "B" and "C" were determined on days -1 and 1 (prior to WR 242,511 administration), and then 1, 2, 4, 6, 8, 24, 48 and 72 hours post test article administration. Serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase levels were determined for these animals on days -1 and 1 (prior to WR 242,511 administration), and then 1, 8 and 72 hours post test article administration.

RESULTS AND DISCUSSION

Mortality

A summary of dosing and mortality data is given in Table 4-1. Animal 5AWL5 of group "A", who had received 7.024 mg/kg of WR 242,511 once per day for four (4) consecutive days, was found dead on Study Day 14. The animal was not necropsied and cause of death was not determined. However, death was considered to have been related to WR 242,511 administration because of increased blood methemoglobin levels and increases in clinical chemistry parameters (see below) observed in this animal prior to death.

Body Weight

Individual body weight data are given in Table 4-2. Fluctuations and/or slight to moderate, but steady, decreases in body weight were observed in all groups. The surviving animal in group "A" (No. 5AWI4) exhibited slight increases (recovery of body weight loss) daily beginning on Study Day 23. The observed decreases in body weight are believed to have been due to test article toxicity. However, the lack of control animals does not completely rule out stress due to numerous bleedings as a contributing factor.

Observations

Incidence of daily observations is given in Table 4-3. The only pharmacotoxic signs noted were decreased activity, anorexia, diarrhea, pale mucous membranes and lack of formed stools in the group "A" animals. All the aforementioned observations were considered associated with the multiple dosing of the test article. All animals in groups "B" and "C" (single dose) were observed as being normal during the study period.

Clinical Laboratory Studies

Methemoglobin Levels: Individual methemoglobin levels following administration of WR 242,511 are given in Table 4-4. Blood methemoglobin levels were increased from baseline values in all groups. The magnitude of the increases appeared to be related to both the dose level and frequency of administration. Animals of groups "B" (single dose at 7.024 mg/kg) and "C" (single dose at 14.048 mg/kg) exhibited noticeable increases beginning at four and six hours post test article administration, respectively. Blood methemoglobin levels were still increasing in these animals 72 hours after WR 242,511 administration. Mean methemoglobin levels in the group "C" animals were approximately 1.5 times greater than those of group "B" animals at the 24, 48 and 72 hour time periods, reflecting the increased dose level for the group "C" animals.

The animals in group "A" (multiple dose; 7.024 mg/kg for 4 consecutive days) exhibited noticeable increases at 24 hours post test article administration. Blood methemoglobin levels were not determined for these animals at 1, 2, 4, 6 and 8 hours post test article administration as they were for animals of groups "B" and "C". At 24 hours post-exposure, mean methemoglobin levels in group "A" animals were equal to those of group "B" animals, but approximately 1.5 times less than those of group "C" animals, again reflecting the increased dose level for group "C". At 48 hours post-exposure, mean methemoglobin levels in group "A" animals were approximately 1.5 times greater than those of group "B" animals (group "A" levels equal to group "C" levels), while by 72 hours post-exposure mean levels for group "A" animals were approximately twice those for group "B" animals and approximately 1.5 times greater than those of group "C" animals. The increased methemoglobin levels in group "A" animals at these time periods reflect the effect of multiple dose administration in these animals.

Blood methemoglobin levels continued to increase in group "A" animals until approximately Study Day 8. Continued and steady decreases in blood methemoglobin levels were present in these animals (group A) beginning approximately Study Day 9. Animal 5AWL5 expired on Study Day 14, but the surviving animal (5AWI4) continued to show decreased methemoglobin levels until study termination (Study Day 33). The blood methemoglobin level of animal 5AWI4 at Study Day 33 was still increased approximately seven times from its baseline value.

Hemoglobin and Hematocrit Levels: Individual hemoglobin and hematocrit data are given in Appendix III. Although there were minor fluctuations (increases and decreases) in hemoglobin and hematocrit levels after WR 242,511 administration from those recorded at baseline in all test groups at various collection times throughout the study, the differences noted were not considered a result of WR 242,511 administration.

Alanine Aminotransferase, Aspartate Aminotransferase, and Alkaline Phosphatase Level: Individual alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (SAP) data are given in Appendix IV. Animal 5AWL5 of group "A" exhibited considerable increases in ALT, AST and SAP at 72 hours, five and 12 days post test article administration when compared to the baseline values. Animal 5AVQ7 of group "C" also exhibited similar increases in these same parameters at 72 hours post test article administration. These increases are indicative of hepatic cellular degeneration, skeletal and cardiac degeneration, and/or obstructive icterus in dogs for ALT, AST and/or SAP, respectively[6]. These increases were most likely a direct result of WR 242,511 administration.

CONCLUSION

Oral administration of WR 242,511 results in increased blood methemoglobin levels in Beagle dogs. The magnitude of the methemoglobin increases appears to be related to both the dose level and frequency of WR 242,511 administration. Methemoglobin clearance from the blood following multiple doses of WR 242,511 takes several days, thus indicating WR 242,511 or its metabolites has a long systemic half-life. Pharmacotoxic signs of decreased activity, anorexia and diarrhea were associated with multiple dosing of WR 242,511. Other toxicity related to WR 242,511 administration included decreased body weight, increased ALT, AST and SAP levels, and death.

Table 4-1

Summary of Dosing and Mortality Data

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Frequency of Dosing	Fate
A	5AWI4 F	7.024	Once daily for 4 consecutive days	S[a]
A	5AWL5 F	7.024	Once daily for 4 consecutive days	D[b]
B	5AVP3 F	7.024	Single dose	S
B	5AWL7 F	7.024	Single dose	S
C	5AWK4 F	14.048	Single dose	S
C	5AVQ7 F	14.048	Single dose	S

[a] S = Sacrificed.

[b] D = Died (Animal expired on Study Day 14).

Table 4-2

Individual Body Weight Data

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Body Weight (kg) on Day 1	2	3	4	5
A	5AWI4 F	7.024[a]	10.1	10.1	9.9	9.7	9.5
	5AWL5 F	7.024[a]	9.8	9.8	9.7	9.6	9.1
B	5AVP3 F	7.024[b]	9.4	9.3	9.1	9.2	-[c]
	5AWL7 F	7.024[b]	9.0	9.0	8.9	8.9	-[c]
C	5AWK4 F	14.048[b]	9.4	9.2	9.0	9.0	-[c]
	5AVQ7 F	14.048[b]	7.6	7.5	7.3	7.1	-[c]

[a] Once daily for 4 consecutive days.

[b] Single dose.

[c] Animal sacrificed on Study Day 4.

Table 4-2

Individual Body Weight Data

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Body Weight (kg) on Day 8	9	10	11	12
A	5AWI4 F	7.024[a]	9.0	8.7	8.5	8.4	8.4
	5AWL5 F	7.024[a]	8.4	8.1	8.0	7.8	7.7

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Body Weight (kg) on Day 15	16	17	18	19
A	5AWI4 F	7.024[a]	8.4	8.4	8.5	8.2	8.3
	5AWL5 F	7.024[a]	-[b]	-	-	-	-

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Body Weight (kg) on Day 22	23	24	25	26
A	5AWI4 F	7.024[a]	8.4	8.8	9.0	8.9	9.1
	5AWL5 F	7.024[a]	-[b]	-	-	-	-

Group	Animal and Sex	Level of WR 242,511 (mg/kg BWt)	Body Weight (kg) on Day 29	30	31	32	33
A	5AWI4 F	7.024[a]	9.0	9.4	9.3	9.2	9.3
	5AWL5 F	7.024[a]	-[b]	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Value not determined; animal expired on Study Day 14.

Table 4-3

Incidence of Daily Observations

Observation	Group: Dosage Level:	Incidence/(Days of Occurrence) A 7.024[a]	B 7.024[b]	C 14.048[b]
Activity decreased		2/2[c] (6-13)	0/2	0/2
		1/1 (14-16)		
Anorexia		2/2 (6-13)	0/2	0/2
		1/1 (14-16)		
Diarrhea		1/2 (6)	0/2	0/2
		2/2 (7,8)		
Mucous membranes, pale		1/2 (6-13)		
		1/1 (14-23)		
No stools		2/2 (9-13)		
		1/1 (14)		
Normal		2/2 (1-5)		
		1/1 (24-33)		

[a] (mg/kg BWt); Once daily for 4 consecutive days.

[b] (mg/kg BWt); Single dose.

[c] Number affected/number observed on day(s) indicated in ( ).

Table 4-4

Individual Methemoglobin Levels Following Administration of WR 242,511

Group	Animal and Sex	Dosage Level (mg/kg BWt)		% Methemoglobin -Time Period (Hour)-										
				0[a]	0[b]	1	2	4	6	8	24	48	72	96
A	5AWI4 F	7.024[c]		0.70	0.67	-[d]	-	-	-	-	19.61	39.84	53.45	60.44
	5AWL5 F	7.024[c]		0.54	0.81	-[d]	-	-	-	-	13.74	29.87	38.06	39.50
			Mean	0.62	0.74	-	-	-	-	-	16.68	34.86	45.76	49.97
B	5AVP3 F	7.024[e]		0.61	0.69	0.88	0.80	1.39	2.65	4.37	15.91	22.16	23.74	-[f]
	5AWL7 F	7.024[e]		0.81	0.54	0.64	0.63	1.71	3.83	6.53	20.00	25.62	26.39	-[f]
			Mean	0.71	0.62	0.76	0.72	1.55	3.24	5.45	17.96	23.89	25.06	-
C	5AWK4 F	14.048[e]		0.72	0.75	0.97	0.86	0.83	2.04	6.71	29.63	41.55	45.45	-[f]
	5AVQ7 F	14.048[e]		0.71	0.77	0.85	0.45	0.90	1.94	5.34	22.67	27.66	29.38	-[f]
			Mean	0.72	0.76	0.91	0.66	0.86	1.99	6.02	26.15	34.60	37.42	-

[a] Baseline value determined on day -1.

[b] Second baseline value determined just prior to dosing on day 1.

[c] Once daily for 4 consecutive days.

[d] Value not determined.

[e] Single dose.

[f] Animal sacrificed on Study Day 4.

Table 4-4

Individual Methemoglobin Levels Following Administration of WR 242,511

Group	Animal and Sex	Dosage Level (mg/kg BWt)		% Methemoglobin -Time Period (Day)- 8	9	10	11	12	15	16	17	18
A	5AWI4 F	7.024[a]		60.93	58.43	56.27	54.25	51.85	41.69	37.84	34.28	30.76
	5AWL5 F	7.024[a]		37.15	35.85	33.73	32.23	29.13	-[b]	-	-	-
			Mean	49.04	47.14	45.00	43.24	40.49	-	-	-	-
B	5AVP3 F	7.024[c]		-[d]	-	-	-	-	-	-	-	-
	5AWL7 F	7.024[c]		-[d]	-	-	-	-	-	-	-	-
C	5AWK4 F	14.048[c]		-[d]	-	-	-	-	-	-	-	-
	5AVQ7 F	14.048[c]		-[d]	-	-	-	-	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Value not determined; animal expired on Study Day 14.

[c] Single dose.

[d] Animal sacrificed on Study Day 4.

Table 4-4

Individual Methemoglobin Levels Following Administration of
WR 242,511

Group	Animal and Sex	Dosage Level (mg/kg BWt)		% Methemoglobin -Time Period (Day)-								
				19	22	23	24	25	26	29	30	33
A	5AWI4 F	7.024[a]		27.45	18.45	16.22	14.32	12.26	10.58	7.59	6.80	4.61
	5AWL5 F	7.024[a]		-[b]	-	-	-	-	-	-	-	-
			Mean	-	-	-	-	-	-	-	-	-
B	5AVP3 F	7.024[c]		-[d]	-	-	-	-	-	-	-	-
	5AWL7 F	7.024[c]		-[d]	-	-	-	-	-	-	-	-
C	5AWK4 F	14.048[c]		-[d]	-	-	-	-	-	-	-	-
	5AVQ7 F	14.048[c]		-[d]	-	-	-	-	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Value not determined; animal expired on Study Day 14.

[c] Single dose.

[d] Animal sacrificed on Study Day 4.

DISCUSSION AND CONCLUSIONS

Much of the research conducted during the past three years centered around measuring in Beagle dogs the degree of protection from lethal intravenous doses of potassium cyanide achieved primarily by increasing blood methemoglobin levels. A number of studies were conducted to evaluate the efficacy of selected compounds as to potential antidotes against cyanide intoxication in an attempt to develop a drug or regimen of drugs which can be used for protection against cyanide intoxication in a battlefield situation where the threat of cyanide exposure is a likely possibility. The research focused on the following areas:

1. Establishing an acute LD_{50} for KCN.

2. Validation of the chemical method for determining methemoglobin in the blood of dogs.

3. The kinetics of methemoglobin formation and disappearance.

4. The effect of pre-existing levels of blood methemoglobin on the ability to resist lethal KCN intoxication.

The majority of the work was conducted using the candidate drugs WR 6026, an 8-aminonquinoline experimental anti-leishmanial drug, and hydroxylamine hydrochloride. The kinetics of methemoglobin formation were also evaluated following oral administration of WR 242,511, an 8-aminoquinoline derivative proposed for use as an anti-malarial drug. Once the kinetics of methemoglobin formation for the various candidate drugs had been determined, the optimum route of administration (intravenous, intramuscular, oral), dosage levels and time of administration (prophylactic vs. therapeutic) which afforded maximum protection against cyanide intoxication were investigated. Attempts were then made to establish a "protective index" against cyanide intoxication for various blood methemoglobin levels. Pyridoxine hydrochloride, pyridoxal 5-phosphate and α-ketoglutarate, compounds which do not induce methemoglobin, were also evaluated for their potential efficacy against lethal cyanide intoxication. Finally, studies using sodium nitrite were conducted to determine its antidotal protection against cyanide intoxication in order to provide a data base so that sodium nitrite could be used as the standard for comparison of other methemoglobin-forming compounds.

In order for a soldier to be "protected" in a battlefield situation where exposure to potentially lethal doses of cyanide is likely, his blood methemoglobin level must 1) already be increased prior to cyanide exposure, or 2) must be increased immediately after exposure, since cyanide is lethal within minutes after exposure, as has been demonstrated in the previously discussed experiments. In either case, the antidote (methemoglobin inducer) should be relatively nontoxic and not interfere with normal behavior. In addition, if the second option is chosen as the preferred method of protection, the antidote must be 1) easily administered, by oneself or, most likely, by a fellow soldier, and 2) effective within minutes after administration.

Both WR 6026 and WR 242,511 were shown to be potent methemoglobin inducers. For both compounds, the magnitude of the methemoglobin increase was related to both the dose level and frequency of administration. Peak blood methemoglobin levels of 19-20% were obtained after four consecutive days of WR

6026 dosing at a dose of 4.83 mg/kg. Methemoglobin levels decreased to approximately 10% five days after the last dose. Blood methemoglobin levels up to nearly 10% were induced by a single administration of WR 6026. Peak blood methemoglobin levels of approximately 50% were obtained after four consecutive days of WR 242,511 administration at a level of 7.024 mg/kg body weight, while methemoglobin levels of approximately 25% were obtained 72 hours after a single dose (7.024 mg/kg) of WR 242,511. A single dose of WR 242,511 at a level of 14.048 mg/kg resulted in peak methemoglobin levels of approximately 37% 72 hours post-dose. Blood methemoglobin levels were elevated for several days following WR 242,511 administration. Single (14.5 mg/kg) or multiple (4.83 mg/kg) doses of WR 6026 were administered without any signs of drug-related toxicity. This was in contrast to WR 242,511 where signs of toxicity, including decreased activity, anorexia, diarrhea, decreased body weight, increased liver enzyme (ALT, AST, SAP) activity levels and death, were noted in one or two animals.

Protective indexes against lethal cyanide intoxication of 3.5 and 1.75 were established for 10-12% and 5-6%, respectively, blood methemoglobin levels induced by oral (capsule) administration of WR 6026. In addition, intravenous administration of sodium thiosulfate in conjunction with prophylactic oral administration of WR 6026 resulted in a noticeable alleviation of cyanide-induced toxic symptoms and an increase in the expected survival rate when thiosulfate was given immediately after cyanide exposure.

Thus, results of the studies with WR 6026, WR 242,511 and sodium thiosulfate indicate that WR 6026 may have some potential use as a cyanide antidote, as long as it is administered prior to cyanide exposure (prophylactic administration). Furthermore, WR 6026 can be taken orally, which would facilitate easy use in a battlefield situation. WR 6026 could possibly be used in conjunction with sodium thiosulfate, although for thiosulfate to be effective, it appears that it must be administered intravenously (as opposed to intramuscularly). Intravenous administration would probably be somewhat difficult in a battlefield situation. Although WR 242,511 is a potent methemoglobin inducer, its associated toxicity, at least at the dose levels used in this study, would preclude its use as a cyanide antidote.

Hydroxylamine hydrochloride was also shown to be a methemoglobin inducer following intramuscular injection in the dog. Intramuscular injection of hydroxylamine resulted in 10-12% blood methemoglobin levels within 5-10 minutes after administration. Hydroxylamine was ineffective as a therapeutic agent against cyanide intoxication, but was effective as a prophylactic agent against KCN intoxication. A protective index of 3.75 was established for 10-12% methemoglobinemia induced by prophylactic intramuscular injection of hydroxylamine hydrochloride. In addition, hydroxylamine hydrochloride could be administered intramuscularly as short as one minute prior to cyanide exposure and protect against lethal cyanide intoxication of up to at least 1.5xLD50. This finding, along with the fact that hydroxylamine administration appeared to cause no toxic side effects, indicates its possible usefulness as a cyanide antidote in a battlefield situation, provided its administered prior to cyanide exposure.

Sodium nitrite was an effective antidote against lethal cyanide intoxication when administered intravenously either ten minutes prior to (prophylactic) or immediately after (therapeutic) cyanide exposure, and when administered intramuscularly ten minutes prior to (prophylactic) cyanide exposure. Sodium nitrite was not, however, an effective antidote when administered via intramuscular injection immediately after (therapeutic)

cyanide exposure. Sodium nitrite is also a potent methemoglobin inducer and its antidotal properties against cyanide intoxication were similar to both WR 6026 and hydroxylamine hydrochloride, although a protective index against cyanide intoxication was not established for sodium nitrite.

Pyridoxal 5-phosphate (PLP), the active cofactor form of pyridoxine hydrochloride (PN; vitamin B6) has been shown to form covalent complexes with cyanide. Keniston et. al.[9] have shown that PLP was able to prolong survival time in rats in which PLP was administered via intraperitoneal injection one minute after a lethal dose of KCN. In our pilot studies investigating the potential effectiveness of PN and PLP in dogs, both compounds were ineffective when administered intravenously ten minutes prior to cyanide exposure. The ineffectiveness of PN may be related to an insufficient time interval to allow for methabolic conversion to the active cofactor form (PLP) prior to cyanide exposure. Further investigation in regard to different routes of administration and possible therapeutic administration in which the antidote is given immediately after exposure to cyanide is needed to adequately evaluate the potential efficacy of PN and PLP against cyanide poisoning.

Another compound which may have some use as an antidote against cyanide intoxication is α-ketoglutanate (α-KG). In our pilot studies with dogs, intramuscular injection of α-KG approximately ten minutes prior to cyanide exposure (prophylactic) did not protect against lethality, whereas cyanide lethality was prevented when α-KG was given intravenously. The effectiveness of oral α-KG administration could not be evaluated because of vomiting of the antidotal solution.

In conclusion, both WR 6026 and hydroxylamine hydrochloride are potent methemoglobin inducers which are capable of protecting against acute cyanide intoxication, either alone or in conjunction with sodium thiosulfate or sodium nitrite. Both antidotes can be given in a form (orally or intramuscular injection) which would be practical in a battlefield situation. Both compounds must, however, be administered prophylactically, i.e., prior to cyanide exposure. Further studies are required to more fully evaluate both the efficacy and toxicity of these compounds prior to human use.

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Appendix I

Clinical Laboratory Procedures

HEMATOLOGY

HEMOGLOBIN

Principle: Whole blood and EDTA are mixed with a modified Drabkin's solution (red blood cell lysing agent + diluting solution) to yield a cyanmethemoglobin reaction in red blood cells as follows:

Hemoglobin + Ferricyanide $\longrightarrow$ Methemoglobin

Methemoglobin + Cyanide $\longrightarrow$ Cyanmethemoglobin

Hemoglobin (oxyhemoglobin, hemoglobin, methemoglobin, carboxyhemoglobin) is then measured spectrophotometrically by a Baker Series 7000 Cell Counter at 540 nm.

Reference: Operator's Manual for Baker Series 7000 Cell Counter.

HEMATOCRIT

Principle: Whole blood with EDTA is diluted in a blood diluting solution. The total number of erythrocytes and the mean corpuscular volume (MCV) of the RBC's is measured using a Baker Series 7000 Cell Counter. MCV is defined as the average volume of the red blood cell expressed in cubic microns (μ^3) (1 micron = 10^{-3} nm). The hematocrit (HCT) is calculated as follows:

$$HCT = RBC \times MCV / 10$$

and is expressed as a percent (%).

Reference: Operator's Manual for Baker Series 7000 Cell Counter.

HEMATOLOGY

TOTAL LEUKOCYTE COUNT

Principle: Whole blood with EDTA is diluted in a blood diluting solution. Total leukocyte count in the suspension is then determined utilizing a Baker 7000 Cell Counter. Results are expressed in cells per cubic millimeter of undiluted whole blood.

Reference: Operator's Manual for Baker Series 7000 Cell Counter.

CLINICAL CHEMISTRY

ALANINE AMINOTRANSFERASE (ALT)

Principle: Serum is added to a buffered mixture containing L-alanine, 2-oxoglutarate, NADH, and lactate dehydrogenase (LDH). The following reactions occur:

2-oxoglutarate + L-alanine $\xrightarrow{ALT}$ pyruvate + glutamate

pyruvate + NADH + H^+ $\xrightarrow{LDH}$ NAD^+ + lactate

The rate of NADH oxidation is spectrophotometrically measured at 340 nm. The results are expressed in International Units per liter of serum. A unit is defined as the oxidation of one micromole of NADH per minute as 30°C.

Reference: Operator's Manual for Multistat III Micro Centrifugal Analyzer (MCA) - Gilford Diagnostics Methodology

CLINICAL CHEMISTRY

ASPARTATE AMINOTRANSFERASE (AST)

Principle: Serum is added to a buffered mixture containing L-aspartate, 2-oxoglutarate, NADH, and malate dehydrogenase (MDH). The following reactions occur:

2-oxoglutarate + L-aspartate $\xrightarrow{AST}$ oxaloacetate + glutamate

oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ NAD^+ + malate

The rate of NADH oxidation is spectrophotometrically measured at 340 nm. The results are expressed in International Units per liter of serum. A unit is defined as the oxidation of one micromole of NADH per minute at 30°C.

Reference: Operator's Manual for Multistat III Micro Centrifugal Analyzer (MCA) - Worthington Statzyme Methodology. Henry, et. al., Amer. J. Clin. Path., 34:381, 1960. Amador and Wacker, Clin. Chem., 8:343, 1962. Trivedi, et al., JAMA, 160:1130, 1956.

CLINICAL CHEMISTRY

ALKALINE PHOSPHATASE

Principle: Serum is added to a buffered (pH 10.2) solution containing p-nitrophenylphosphate. The following reactions occur:

p-nitrophenylphosphate + H_2O $\xrightarrow{\text{alk. phos.}}$ p-nitrophenol + phosphate (H_3PO_4)

p-nitrophenol exhibits a yellow color in the alkaline medium The rate of p-nitrophenol formation is measured spectrophotometrically at 405 nm. Results are expressed in International Units per liter of serum. A unit is defined as the formation of one micromole of p-nitrophenol per minute at 30°C and pH 10.4.

Reference: Operator's Manual for Multistat III Micro Centrifugal Analyzer (MCA) - Gilford Diagnostics Methodology

Appendix II

Individual Hematology Data

(Part 2 - Evaluation of the Prophylactic Effect of Pyridoxine Hydrochloride and Pyridoxal 5-Phosphate Against Potassium Cyanide Intoxication Following Intravenous Injection - Pilot Study)

Individual Hematology Data

Group: A

Pyridoxine Hydrochloride (mg/kg): 300

Animal and Sex	Pretest			Predose			5 Min.		10 Min.		15 Min.		30 Min.	
	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)
4AVX4 F	9.2	17.0	49.6	8.6	16.1	46.9	15.7	46.1	15.2	44.0	17.2	50.8	-	-
5AWJ4 F	12.0	17.9	50.9	11.0	15.5	43.6	16.4	48.7	15.3	43.5	-	-	-	-
5AVN7 F	13.9	16.6	47.9	13.2	15.8	45.2	-	-	-	-	-	-	-	-

Individual Hematology Data

Group: B

Pyridoxal 5-Phosphate (mg/kg): 300

Animal and Sex	Pretest			Predose			5 Min.		10 Min.		15 Min.		30 Min.	
	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)
5AVK5 F	10.1	16.4	44.3	10.9	14.6	41.7	-	-	-	-	-	-	-	-
5AWG2 F	10.4	18.1	53.4	14.1	16.0	45.8	17.3	48.7	16.8	48.2	-	-	-	-
5AWI3 F	11.9	17.6	51.0	14.3	15.9	45.2	17.9	52.3	18.8	54.2	-	-	17.7	50.6

Individual Hematology Data

Group: C

Pyridoxal 5-Phosphate (mg/kg): 300

Animal and Sex	Pretest			Predose			5 Min.		10 Min.		15 Min.		30 Min.	
	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	WBC ($x10^3/\mu l$)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)	Hb (gm/dl)	Hct (%)
5AVX3 F	7.9	18.8	52.7	-	-	-	-	-	-	-	-	-	-	-

Appendix III

Individual Hematology Data

(Part 4 - Evaluation of the Kinetics of Methemoglobin Formation

Induced By Oral Administration of WR 242,511)

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day -1			0 Hour			1 Hour			2 Hours			4 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWI4 F	18.3	55.1	0.70	17.1	50.4	0.67	-[b]	-	-	-	-	-	-	-	-
5AWL5 F	20.4	59.7	0.54	17.1	49.9	0.81	-[b]	-	-	-	-	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Value not determined.

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	6 Hours			8 Hours			24 Hours			48 Hours			72 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWI4 F	-[b]	-	-	-	-	-	17.0	50.7	19.61	17.4	51.3	39.84	16.3	47.3	53.45
5AWL5 F	-[b]	-	-	-	-	-	16.8	51.7	13.74	17.3	49.4	29.87	16.5	47.1	38.06

[a] Once daily for 4 consecutive days.

[b] Value not determined.

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	96 Hours			Day 8			Day 9			Day 10			Day 11		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWI4 F	16.7	48.2	60.44	17.0	47.0	60.93	17.2	49.2	58.43	17.0	50.6	56.27	17.1	50.5	54.25
5AWL5 F	17.0	48.9	39.50	17.3	49.3	37.15	18.2	53.8	35.85	18.1	55.0	33.73	19.3	57.0	32.23

[a] Once daily for 4 consecutive days.

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day 12			Day 15			Day 16			Day 17			Day 18		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWI4 F	16.6	49.0	51.85	17.1	52.0	41.69	16.8	49.8	37.84	16.4	49.1	34.28	15.7	46.6	30.76
5AWL5 F	20.7	62.1	29.13	-[b]	-	-	-	-	-	-	-	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Animal expired on Study Day 14.

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day 19			Day 22			Day 23			Day 24			Day 25		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWI4 F	15.8	47.9	27.45	15.4	47.2	18.45	16.5	50.6	16.22	16.6	47.5	14.32	16.8	49.2	12.26
5AWL5 F	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Once daily for 4 consecutive days.

[b] Animal expired on Study Day 14.

Individual Hematology Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day 26			Day 29			Day 30			Day 33					
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWT4 F	16.1	49.8	10.58	15.2	44.4	7.59	16.2	50.1	6.80	16.4	49.5	4.61			
5AWL5 F	-[b]	-	-	-	-	-	-	-	-	-	-	-			

[a] Once daily for 4 consecutive days.

[b] Animal expired on Study Day 14.

Individual Hematology Data

Group: B

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day -1			0 Hour			1 Hour			2 Hours			4 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AVP3 F	17.9	52.8	0.61	16.6	47.5	0.69	17.3	49.5	0.88	17.4	49.6	0.80	17.3	49.5	1.39
5AWL7 F	18.2	53.6	0.81	16.2	48.4	0.54	17.5	51.3	0.64	17.7	51.2	0.63	17.5	51.1	1.71

[a] Single dose.

Individual Hematology Data

Group: B

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	6 Hours			8 Hours			24 Hours			48 Hours			72 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AVP3 F	17.3	50.2	2.65	17.6	52.3	4.37	15.9	45.6	15.91	17.1	49.2	22.16	15.9	46.3	23.74
5AWL7 F	17.9	51.8	3.83	17.8	54.0	6.53	14.5	43.0	20.00	16.0	46.3	25.62	15.3	45.8	26.39

[a] Single dose.

Individual Hematology Data

Group: C

Level of WR 242,511 (mg/kg BWt): 14.048[a]

Animal and Sex	Day -1			0 Hour			1 Hour			2 Hours			4 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWK4 F	19.0	55.3	0.72	16.8	48.8	0.75	17.6	52.1	0.97	17.4	50.8	0.86	17.9	52.1	0.83
5AVQ7 F	19.2	56.3	0.71	17.8	52.0	0.77	19.4	56.2	0.85	19.4	56.1	0.45	19.6	55.4	0.90

[a] Single dose.

Individual Hematology Data

Group: C

Level of WR 242,511 (mg/kg BWt): 14.048[a]

Animal and Sex	6 Hours			8 Hours			24 Hours			48 Hours			72 Hours		
	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)	Hb (gm/dl)	Hct (%)	Methgb (%)
5AWK4 F	18.7	53.8	2.04	17.8	51.5	6.71	16.8	48.5	29.63	17.5	50.0	41.55	17.1	50.1	45.45
5AVQ7 F	19.0	55.0	1.94	18.6	53.4	5.34	16.6	48.7	22.67	17.6	51.5	27.66	19.2	55.5	29.38

[a] Single dose.

Appendix IV

Individual Clinical Chemistry Data

(Part 4 - Evaluation of the Kinetics of Methemoglobin Formation

Induced By Oral Administration of WR 242,511)

Individual Clinical Chemistry Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day -1			0 Hour			1 Hour			8 Hours			24 Hours		
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AWI4 F	24.2	20.0	59.3	24.5	17.2	55.9	-[b]	-	-	-	-	-	24.4	16.2	54.9
5AWL5 F	19.7	25.7	69.3	16.3	19.2	63.1	-[b]	-	-	-	-	-	20.0	18.2	63.4

[a] Once daily for 4 consecutive days.

[b] Value not determined.

Individual Clinical Chemistry Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	48 Hours			72 Hours			Day 5			Day 12			Day 19		
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AWI4 F	24.0	28.6	64.7	27.6	17.8	67.4	35.1	15.0	72.8	28.1	16.9	77.4	19.2	14.9	48.6
5AWL5 F	25.6	18.5	57.3	699.3	257.4	91.0	2386.0	400.8	172.3	298.3	16.4	140.0	-[b]	-	-

[a] Once daily for 4 consecutive days.

[b] Animal expired on Study Day 14.

Individual Clinical Chemistry Data

Group: A

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day 26														
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AW14 F	16.2	17.5	50.5												
5AW15 F	-[b]	-	-												

[a] Once daily for 4 consecutive days.

[b] Animal expired on Study Day 14.

Individual Clinical Chemistry Data

Group: B

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	Day -1			0 Hour			1 Hour			8 Hours			24 Hours		
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AVP3 F	21.8	23.8	56.3	21.5	24.5	55.5	21.1	26.1	53.7	22.2	25.2	57.2	-[b]	-	-
5AWL7 F	18.3	18.2	59.8	15.5	13.6	59.2	16.5	17.3	61.3	18.1	16.3	61.6	-[b]	-	-

[a] Single dose.

[b] Value not determined.

Individual Clinical Chemistry Data

Group: B

Level of WR 242,511 (mg/kg BWt): 7.024[a]

Animal and Sex	48 Hours			72 Hours											
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AVP3 F	-[b]	-	-	22.8	25.5	59.0									
5AWL7 F	-[b]	-	-	17.6	14.5	64.2									

[a] Single dose.

[b] Value not determined.

Individual Clinical Chemistry Data

Group: C

Level of WR 242,511 (mg/kg BWt): 14.048[a]

Animal and Sex	Day -1			0 Hour			1 Hour			8 Hours			24 Hours		
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AWK4 F	22.0	22.8	50.6	22.3	26.4	52.6	24.7	30.9	58.7	22.8	21.0	61.3	-[b]	-	-
5AVQ7 F	26.0	22.2	57.5	24.6	21.6	54.4	29.4	27.0	58.8	28.5	20.6	55.3	-[b]	-	-

[a] Single dose.

[b] Value not determined.

Individual Clinical Chemistry Data

Group: C

Level of WR 242,511 (mg/kg BWt): 14.048[a]

Animal and Sex	48 Hours			72 Hours											
	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)	ALT (IU/L)	AST (IU/L)	SAP (mg/dl)
5AWK4 F	-[b]	-	-	37.9	26.0	66.9									
5AVQ7 F	-[b]	-	-	1284.0	151.9	75.2									

[a] Single dose.

[b] Value not determined.

Appendix V

Quality Assurance Unit Statement

This final report was reviewed as required by Good Laboratory Practice Regulations for non-clinical laboratory studies, 21 CFR Part 58 and the study protocol. Inspections were accomplished as noted, and reported to the study director and management immediately following their completion. Based on these inspections and the review of the report, this study was conducted and reported in conformance with the Good Laboratory Practice regulations.

Frederick D. Paul 5/7/87

Frederick F. Paul,
Quality Assurance Unit

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STUDY NO. 7583. QAU INSPECTION SCHEDULE (ACUTES)

TYPE TEST	INSPECTION DATES DATE RESULTS REPORTED TO MANAGEMENT			
Dog	8/29/86 SEA	9/2/86 DHP	9/10/86 SEA	9/18/86 SEA
	9/2/86 SEA	9/2/86 DHP	9/10/86 SEA	9/18/86 SEA
	9/26/86 SEA	9/30/86 DHP	10/16/86 DHP	10/31/86 DHP
	9/29/86 SEA	9/30/86 DHP	10/17/86 DHP	10/31/86 DHP
	11/7/86 DHP	11/12/86 DHP	12/2/86 DHP	12/10/86 DHP
	11/7/86 DHP	11/12/86 DHP	12/2/86 DHP	12/10/86 DHP
	12/18/86 DHP			
	12/18/86 DHP			